

IN VACUO GLYCATION OF PROTEINS**FIELD OF THE INVENTION**

The invention relates to a method of glycating proteins or material containing at least one amino group.

5 BACKGROUND OF INVENTION

Glycoproteins are involved in a wide variety of multivalent interactions that are of physiological importance. The many potential roles of carbohydrates covalently attached to proteins are of interest in theoretical structure-function studies and practical applications. In particular, applications such as increasing the stability and
10 solubility of proteins (Sundaram, P.V. and Venkatesh, R., 1998, *Protein Eng.*, **11**, 699-705, Ed.), (Aoki, T., Hiidome, Y., Kitahata, K., Sugimoto, Y., Ibrahim, H.R. and Kato, Y., 1999, *Food Res. Int.*, **32**, 129-133, Ed.) and the development of vaccines (Mammen, M., Choi, S.-K. and Whitesides, G., 1998, *Angew. Chem. Int. Ed.*, **37**, 2754-2794, Ed.), (Wong, S.Y., 1995, *Current Opin. Struct. Biol.*, **5**, 559-
15 604, Ed.), (Roy, R., 1996, *Current Opin. Struct. Biol.*, **6**, 692-702, Ed.) have spurred efforts to discover efficient methods of chemical attachment of carbohydrate to proteins, i.e. glycation. Protein glycation is often carried out under aqueous conditions where the reaction is unfavorable due to the fact that water is a product. Current aqueous glycation methods also require relatively large amounts of protein
20 and carbohydrate (Yeboah, F.K., Alli, I. and Yaylayan, V.A., 1999, *J. Agric. Food Chem.*, **47**, 3164-3172, Ed.), (Roy, R., Katzenellenbogen, E. and Jennings, H.J., 1984, *Can. J. Biochem. Cell Biol.*, **62**, 270-275, Ed.), and the extent of glycation is very difficult to control (Wrodnigg, T.M. and Eder, B., 2001, *Glycoscience*, **215**, 115-152, Stutz, A.E., Ed., Springer-Verlag). Another significant drawback is that
25 the desired glycation product, cyclic ketoamine, is contaminated with advanced glycation products (Wrodnigg, T.M. and Eder, B., 2001, *Glycoscience*, **215**, 115-152, Stutz, A.E., Ed., Springer-Verlag), (Yaylayan, V.A. and Huyghues-Despointes, A., 1994, *Crit. Rev. Food Sci. Nutrition*, **34**, 321-369, Ed.) i.e. Maillard browning reaction.

An effective method of increasing the efficiency of a reaction is by the removal of a product. The first step in the glycation of an amino group is believed to be the reaction of a deprotonated amine with the aldehyde group of a reducing sugar yielding water and a Schiff base as products (Yaylayan, V.A. and Huyghues-Despointes, A., 1994, *Crit. Rev. Food Sci. Nutrition*, **34**, 321-369, Ed.), (Wrodnigg, T.M. and Eder, B., 2001, *Glycoscience*, **215**, 115-152, Stutz, A.E., Ed., Springer-Verlag). In aqueous media, formation of the Schiff base is reversible, and *in situ* reductive alkylation of the Schiff base forming a stable derivative has been employed to achieve efficient glycation (Cayot, P., Roullier, L. and Tainturier, G., 1999, *J. of Agric. & Food Chem.*, **47**, 1915-1923, Ed.), (Wrodnigg, T.M. and Eder, B., 2001, *Glycoscience*, **215**, 115-152, Stutz, A.E., Ed., Springer-Verlag). Theoretically, another way of promoting the glycation reaction is by the removal of the water but there is no obvious experimental approach for such a strategy under aqueous conditions. Water could be removed by carrying out the reaction in the dry state under vacuum. Dry state glycation has been attempted under a variety of conditions (Boratynski, J. and Roy, R., 1998, *Glycoconjugate J.*, **15**, 131-138, Ed.), (Boratynski, J., 1998, *Biotechnol. Tech.*, **12**, 707-710, Ed.), (Quan, C.P., Wu, S., Dasovich, N., Hsu, C., Patapoff, T. and Canova-Davis, E., 1999, *Anal. Chem.*, **71**, 4445-4454, Ed.), (Morgan, F., Leonil, J., Molle, D. and Bouhallab, S., 1999, *J. Agric. Food Chem.*, **47**, 83-91, Ed.), (Yeboah, F.K., Alli, I., 1999, *J. Agric. Food Chem.*, **47**, 3164-3172, Ed.), (French, S.J., Harper, W.J., Kleinholz, N.M., Jones, R.B. and Green-Church, K.B., 2002, *J. Agric. Food Chem.*, **50**, 820-823, Ed.) but a common observation is that many glycation products are observed and the mechanism of their formation is unclear. None of these studies have used a vacuum to promote the glycation reaction by the removal of water or to prevent the formation of advanced glycation end products.

Amino groups in dry proteins are present in their protonated form, and for glycation to take place, the reaction would have to involve these protonated amino groups. On the basis of current theory, a protonated amino group in solution does not react with the aldehyde form of a reducing sugar. Furthermore, there is no known theory that

- predicts that if a mixture of a protein and reducing sugar in the dry state were subjected to a vacuum that a water-stable glycated derivative would be formed. The fact that extensive glycation of proteins does occur in the lyophilized state under vacuum with heating demonstrates that the protonated amino group does indeed
- 5 react. Therefore, there are two novel theoretical features to the discovery that lyophilized proteins can be efficiently glycated *in vacuo* in the dry state: 1. A protonated amino group will react with a sugar aldehyde group *in vacuo*. 2. A ketoamine derivative is formed which does not rapidly revert to the free amine and sugar when placed in aqueous solution.
- 10 Covalent cross-linking of proteins is a major tool for the study of structure-function relationships in proteins and has many practical applications (Fancy, D.A., 2001, *Curr. Opin. Chem. Biol.*, 4, 28-33, Ed.), (Phizicky, E.M., 1995, *Microbiol. Rev.*, 59, 94-123, Ed.), (Lundblad, R., 1994, *Techniques in Protein Modification*, 249-261, Ed., CRC Press). Homo-bifunctional reagents with variable spacing between the
- 15 reactive groups have been widely used to achieve such cross-links (Lundblad, R., 1994, *Techniques in Protein Modification*, 249-261, Ed., CRC Press). To our knowledge, reducing sugars have never been used as bifunctional reagents for the cross-linking of proteins. However, the discovery of the facile glycation that occurs *in vacuo* indicates that a reagent with two or more reducing sugars with variable
- 20 spacing could be used to covalently cross-link proteins in the lyophilized state. The ideal number of spacer units separating the reducing sugars depends on the protein or proteins being cross-linked. In general, the ideal number of units will likely be less than 10 but in some applications could be much greater. The cross-linking methodology would be identical to the *in vacuo* glycation with reducing sugars.
- 25 *In vacuo* glycation is easier to carry out than aqueous glycation. It requires only co-lyophilization of appropriate amounts of protein and reducing sugar followed by incubation at an elevated temperature under vacuum. In addition, *in vacuo* glycation has several other significant technical advantages.
1. The procedure can be carried out using a wide range of protein and/or
- 30 carbohydrate quantities, viz. gram to picogram quantities.

2. The protein can be lyophilized at a pH value where it retains its native structure and biological activity.
3. The extent of glycation is easily controlled either by adjusting the protein/carbohydrate ratio or by the addition of excipients.
- 5 4. Elevated temperatures can be used to increase the rate of glycation without structural damage to the protein or carbohydrate.
5. With proteins, no contaminating advanced glycation products (Maillard browning reaction) are observed. Only the ketoamine derivative is observed.
6. Complex carbohydrates or compounds containing two or more reducing
10 sugars can be used to cross-link proteins.

There is therefore a need for a facile method of glycating proteins.

SUMMARY OF INVENTION

The invention provides a method of glycating proteins and of achieving covalent cross-linking of proteins.

- 15 The invention is the glycation (non-enzymatic covalent attachment of sugars) of proteins by the use of an *in vacuo* reaction of a reducing sugar with one or more amino groups to form a stable ketoamine derivative(s). Reducing monosaccharides or more complex carbohydrates or compounds containing at least one reducing sugar can be employed. The extent of glycation can be controlled by incubation
20 temperature, protein/reducing sugar ratio, addition of excipients (e.g. trehalose) and pH of lyophilization. The *in vacuo* glycation reaction can be used to achieve covalent cross-linking of proteins by the use of carbohydrates or compounds containing two or more reducing sugars.

- 25 According to one aspect of the invention there is provided a method of glycating a protein comprising the following steps:

- combining a quantity of one of a reducing sugar and a reducing polysaccharide with the protein in a solution
 - lyophilizing the solution to produce a lyophilized sample;
 - placing said lyophilized sample under vacuum; and
- 5 • heating said lyophilized sample under vacuum.

According to another aspect of the invention, there is provided a method of glycating a protein comprising the following steps:

- combining a linker including at least two reducing sugars with the protein in a solution;
- 10 • lyophilizing the solution to produce a lyophilized sample;
- placing said lyophilized sample under vacuum; and
 - heating said lyophilized sample under vacuum.

According to another aspect of the invention there is provided a method of glycating a protein comprising the following steps:

- 15 • combining a linker including at least two reducing sugars with the at least two proteins in a solution;
- lyophilizing the solution to produce a lyophilized sample;
 - placing said lyophilized sample under vacuum; and
 - heating said lyophilized sample under vacuum.

According to yet another aspect of the invention there is provided a method of glyrating a protein comprising the following steps:

- combining a linker including at least two reducing sugars with a protein
- lyophilizing the solution to produce a lyophilized sample;
- 5 • placing said lyophilized sample under vacuum;
- heating said lyophilized sample under vacuum.
- isolating the glyrated monomeric product
- combining the glyrated monomeric product with a protein
- lyophilizing the solution to produce a lyophilized sample;
- 10 • placing said lyophilized sample under vacuum; and
- heating said lyophilized sample under vacuum.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a Solution ^{13}C NMR of glyrated RNase with $^{13}\text{C}_1\text{-D-glucose}$.
Inset: Expanded region of the predominant peak.

- 15 Figure 2 is a plot showing time course and effect of pH of lyophilization on *in vacuo* glyration. RNase with a trace of ^{14}C -[U]-glucose was lyophilized at pH 6.5 and pH 10, heated incubated at 65°C for up to 24 hours.

Figure 3 is a plot showing extent of *in vacuo* glyration of lyophilized human hemoglobin incubated 65°C , based on 48 possible glyration sites.

Figure 4 is a plot showing A - 4,7,10-trioxa-1,13-tridecanediglucuronamide (TTDG)
B – polypropyleneiminetetraglucuronamide dendrimer (PTGD).

Figure 5 is a SDS-PAGE of human hemoglobin (Hb) co-lyophilized with increasing concentrations of the homobifunctional cross-linker TTDG at pH 10.0 (adjusted with
5 1 N NaOH) A) with trehalose (0.6 mg/mg Hb), B) without trehalose, and heated at 60°C for 20 hours under vacuum. Concentrations in each lane expressed as moles TTDG/mole Hb: Lane 1- MW markers; Lane 2 – 0; Lane 3 – 0.2; Lane 4 – 1; Lane 5 – 2; Lane 6 – 10; Lane 7 - 20.

Figure 6 is a SDS-PAGE showing cross-linking between human hemoglobin (Hb)
10 and increasing amounts of the branched cross-linker PTGD co- lyophilized at pH 5.8 (unadjusted) and heated under vacuum at 65°C for 19 hours. Concentrations in each lane expressed as moles PTGD/mole Hb: Lane 1: 0; Lane 2 – 0.2; Lane 3 – 0.5; Lane 4 – 0.7; Lane 5 – 1; Lane 6 – 1.2; Lane 7 - 1.5; Lane 8 – MW markers.

DETAILED DESCRIPTION OF INVENTION

15 Materials and Methods

Materials

Ribonuclease A (Sigma R-4875) from bovine pancreas and human hemoglobin (Sigma H-7379) were purchased from Sigma Chemical Company and used without further purification.

20 D-[U-¹⁴C] glucose (310 mCi/mmol), 50 µCi in 250 µl aqueous solution containing 3% ethanol was purchased from Amersham Pharmacia Biotech. D-Glucose-1-¹³C, 99 atom % ¹³C, was purchased from Sigma Chemical Company. All other chemicals, reagents and solvents used were high purity preparations obtained from commercial sources.

25 *In vacuo* glycation of lyophilized proteins

Typically, protein was added to a glucose solution for a final ratio of 5:1 or 10:1 protein/glucose (w/w). The pH of the solution was adjusted with 1M HCl or NaOH, via micro-syringe, to the desired pH (6.5 to 10). Aliquots of protein/reducing sugar solutions were transferred to borosilicate test tubes and frozen by immersing in liquid nitrogen. The samples were subsequently lyophilized to room temperature. The tubes, containing the lyophilized protein/reducing sugar mixture, were narrowed and sealed under vacuum ($1-50 \times 10^{-3}$ torr) with an oxygen-enriched flame, placed in an oven at 65°C and left to react for a controlled period of time (2 to 24 hours).

***In vacuo* glycation with D-[U-¹⁴C] glucose**

Trace radio-glycation of RNase was prepared by dissolving RNase (20 mg, 1400 nmol) in dH₂O (10 ml), adding D-[U-¹⁴C] glucose solution (20 µl, 13 nmol; see above), adjusting the pH with 1M NaOH (pH 6.5 or 10) and lyophilizing as described above.

A hemoglobin/glucose solution (10:1 w/w) was prepared containing D-[U-¹⁴C] glucose 37.5 nCi per mg of protein. After application of the *in vacuo* reaction procedure, samples were dissolved in 10% glucose solution to displace un-reacted D-[U-¹⁴C]glucose and extensively dialyzed against dH₂O.

Aliquots of the protein/reducing sugar solution (50 µl) were transferred to scintillation vials before and after dialysis, scintillation cocktail (5 ml) (Aquasol-2, DuPont) was added and the samples were counted for 10 minutes on a Beckman LS 6500 multi-purpose scintillation counter using the ¹⁴C window. Protein amount was normalized by recording absorbance at 280nm on a Pharmacia Biotech Ultraspec 2000 UV/Vis spectrophotometer with dH₂O as blank.

***In vacuo* glycation with D-[1-¹³C] glucose**

RNase (106.54 mg) was dissolved in 50 ml dH₂O in a 250 ml RB flask, D-glucose-1-¹³C (10.60 mg) was added to the protein solution, the pH was raised to 10 with 1 M NaOH and the solution was frozen/shelled in N₂(ℓ) and lyophilized.

The lyophilized protein: reducing sugar mixture was transferred to a borosilicate hydrolysis tube and glycated as above for 24 hours.

NMR Spectroscopic Analysis

5 RNAse glycated with D-Glucose-1-¹³C was extensively dialysed against dH₂O (MWCO 3500), lyophilized and dissolved in ²H₂O (99.9 atom % ²H, Cambridge Isotope Laboratories) and the ¹³C-NMR spectrum was acquired on a Bruker spectrometer operating at 9.4 Tesla (¹³C, 100.6 MHz) for 600 scans using the DEPT 135 pulse sequence (Sanders and Hunter, 1987).

Synthesis of Sugar Cross-linkers

10 Bifunctional cross-linkers containing reducing sugars separated by a spacer (Figure 4a) were synthesized from glucose derivatives. Glucosamine or glucuronic acid are glucose derivatives which contain functional groups through which the spacer can be coupled, a carboxylic group in the former and an amino group in the latter. Bis acids and amines of vary lengths were used to furnish the linear spacers and were
15 coupled to the appropriate glucose derivative using a water-soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide). Branched cross-linkers were synthesized from glucuronic acid and polypropyleneimine dendrimer (Generation 1.0) (Figure 4b) using O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HATU) as coupling agent.

20 *In Vacuo* Cross-linking Reaction Method

Typically, a stock solution of accurately weighed protein/mL of distilled H₂O was prepared and, depending on the experiment, the pH was adjusted with either 1N NaOH or 1N HCl. Aliquots of the protein stock solution of a minimum volume of 500 µL to yield between 2-5 mg protein/tube, were transferred to 13 x 100 mm
25 borosilicate glass disposable culture tubes to which varying aliquots of a solution of cross-linker dissolved in distilled H₂O were added. The tubes were left to stand for approximately 10 – 15 minutes and were then flash frozen and lyophilized. The tubes were then sealed under vacuum using an oxygen-enriched flame and incubated

at 60°C - 85°C for a period of 12-18 hours. The protein was reconstituted with 0.5 mL of distilled H₂O and analyzed by SDS-PAGE, HPLC or FPLC.

SDS-PAGE Analysis of Cross-linked Proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed using a
5 Bio-Rad Mini-PROTEAN II dual slab cell apparatus. All reagents and molecular weight markers were purchased from BioRad or Sigma, and were electrophoresis grade quality (buffers were prepared as required using dH₂O). Approximately 10 µg of protein/well was loaded onto a 12% polyacrylamide gel and electrophoresis was conducted at a constant current of 20-25 mA/gel (with a voltage gradient of 70-150
10 mV) under discontinuous gel conditions. Coomassie Brilliant Blue G250 was used to stain the protein bands in the gel following electrophoresis.

RESULTS AND DISCUSSION

Solution ¹³C NMR of glycated RNase with ¹³C₁- D -glucose reveals that only one product is present. The single resonance peak, located at 53.2 ppm in Figure 1,
15 corresponds to the cyclic ketoamine adduct (Neglia, C.I., Cohen, H.J., Garber, A.R., Ellis, P.D., Thorpe, S.R. and Baynes, J.W., 1983, *J. Biol. Chem.*, **258**, 14279-14283, Ed.). It is particularly notable that no advanced glycation end-products (browning reaction) are present as is the case in conventional non-reductive glycation procedures (Yaylayan, V.A. and Huyghues-Despointes, A., 1994, *Crit. Rev. Food*
20 *Sci. Nutrition*, **34**, 321-369, Ed.). Conventional glycation procedures require the use of a reducing agent such as sodium borohydride to form a stable amino alcohol derivative and prevent the production of advanced glycation end-products. Another advantage of the *in vacuo* glycation procedure is that no additional reaction is required to stabilize the glycation product as it yields a stable ketoamine product
25 directly.

D-[U-¹⁴C] glucose (310 mCi/mmol) was used directly as supplied without addition of any unlabeled glucose to determine the rate of glycation using the *in vacuo* procedure. Figure 2 shows that the rate of incorporation at pH 6.5 and pH 10 is very

similar and is essentially complete after 12 hours with incubation at 65°C. Figure 3 shows that within experimental error all the amino groups of hemoglobin can effectively be glycosylated by the *in vacuo* glycation procedure.

5 The accepted mechanism for the glycation in water involves the nucleophilic attack of a protein's amino group on the open chain form of the reducing sugar forming a Schiff base which undergoes Amadori rearrangement to form the ketoamine derivative. As lysine ϵ -amino groups in proteins normally have ionization constants of 10.5 to 11, very little glycation of amino groups would be expected below pH 10.5 where the protonated form of the amino group predominates. However, the results in Figure 3 show that the protonated form of the amino group is readily glycosylated by the *in vacuo* procedure. The mechanism by which this glycation occurs has not been established but it is clearly different from that postulated for glycation in solution.

15 The *in vacuo* glycation methodology has been used to develop a new class of protein cross-linking reagents, viz. linear bifunctional and multifunctional branched glyco-crosslinkers. Two such cross-linking reagents have been synthesized, 4, 7, 10-trioxa-1,13-tridecanediglucuronamide (TTDG) (Figure 4a) and polypropyleneimine tetraglucuronamide dendrimer (PTGD) (Figure 4b). Figure 5 shows cross-linking of human hemoglobin with increasing amounts of the bifunctional glyco-crosslinker TTDG using the *in vacuo* glycation methodology. Substantial amounts of dimer and higher oligomers of cross-linked hemoglobin are clearly visible (Figure 5a) with SDS-PAGE, as well as some insoluble, very highly cross-linked material that does not enter the gel. Figure 5b shows the effect of the addition of an excipient, trehalose which promotes the formation of soluble cross-linked oligomers. The glycation procedure with the branched glyco-crosslinker PTGD also yields substantial amounts of dimer and higher oligomers of hemoglobin (Figure 6).

CONCLUSIONS

The results obtained in the present study show that facile glycation of proteins can be achieved by an *in vacuo* reaction of the protein with a reducing sugar or compounds containing one or more reducing sugars. The *in vacuo* glycation
5 procedure can be used with linear bifunctional and multifunctional branched glyco-derivatives containing reducing sugars to achieve facile covalent cross-linking of proteins.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various
10 modifications may be made without deviating from the spirit and scope of the invention.

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